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Microbial Degradation of Nitrogen, Oxygen and Sulfur Heterocyclic Compounds under Anaerobic Conditions: Studies with Aquifer Samples

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16. ABSTRACT

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MICROBIAL DEGRADATION OF NITROGEN, OXYGEN AND SULFUR HETEROCYCLIC COMPOUNDS UNDER ANAEROBIC CONDITIONS: STUDIES WITH AQUIFER SAMPLES

ELMAR P. KUHN and JOSEPH M. SUFLITA*
The University of Oklahoma, Department of Botany and Microbiology, Norman, Oklahoma 73019

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Abstract—The potential for anaerobic biodegradation of 12 heterocyclic model compounds was studied. Nine of the model compounds were biotransformed in aquifer slurries under sulfate-reducing or methanogenic conditions. The nitrogen and oxygen heterocyclic compounds were more susceptible to anaerobic biodegradation than those compounds containing a sulfur heteroatom. Carboxy-substituted compounds were anaerobically metabolized more readily than unsubstituted or methylated analogues. In methanogenic incubations, 47 to 84% of the expected amount of carbon in pyridine, 4-picoline, nicotinic acid and 2-thiophene carboxylic acid was recovered as methane. In contrast, only small amounts of methane were detected in aquifer slurries amended with compounds containing an oxygen heteroatom, even though a decrease in the parent substrate concentration occurred. Pyridine, 2-picoline and 4-picoline were biotransformed within three months under sulfate-reducing conditions. However, longer incubation times were required for the degradation of these substrates in methanogenic aquifer slurries. A literature survey reveals the widespread contamination of ground waters with heterocyclic compounds from waste management practice and fossil-fuel-related industries.

Keywords—Anaerobic biodegradation Pollutant metabolism Aquifers Groundwater Heterocyclic compounds Sulfate reduction Methanogenesis

INTRODUCTION

Two-thirds of the estimated 4 million known organic compounds are heterocyclic chemicals [1]. Such chemicals are widely used as pharmaceuticals, pesticides, explosives, dyes, clearing and sweetening agents. Petroleum and coals also contain a variety of sulfur heterocyclic compounds [2], and thermal conversion of coal and oil shale to synthetic fuels can generate nitrogen-containing azaarenes [3,4]. Some heterocyclic pollutants like the chlorinated dibenzofurans, dioxins and aza hydrocarbons are toxic xenobiotic chemicals of environmental concern [5–7]. Naturally occurring heterocyclic compounds such as nucleotides, vitamins, coenzymes, heme and chlorophyll are not of toxicological concern, but some mycotoxins and alkaloids can be potent carcinogens or poisons [1,8].

Heterocyclic aromatic structures are more polar than their homocyclic analogues and possess lower octanol/water partition coefficients (K_{ow}). For instance, *o*-picoline has a log K_{ow} of 1.06 [9], whereas toluene has a K_{ow} value more than two orders of magnitude higher [10]. The low K_{ow} values are associated with high water solubilities and weak sorption of the chemicals to soil and aquifer organic material [11,12]. Therefore, many heterocyclic pollutants are rapidly transported to the terrestrial subsurface and can contaminate vast ground water reservoirs [13,14].

Sources of ground water contamination with such chemicals include wood preserving facilities, coal gasification sites, oil shale processing plants, hazardous waste sites, municipal landfills and river infiltrates polluted by sewage effluents (Table 1). Several heterocyclic pesticides have also been found in U.S. aquifers (Table 1 of [15]), and in some cases the concentration of these pollutants in ground waters approaches the tolerance limits set for foods [16]. The ground water pollutants include nitrogen, oxygen and sulfur heterocyclic compounds, most of which are aromatic, such as the pyridine, furan and thiophene derivatives (Table 1). However, saturated heterocyclic chemicals like 1,4-dioxane or

*To whom correspondence may be addressed.
The current address of E. P. Kuhn is Kantonales Laboratorium, Kunsthausweg 24, CH-5000 Aarau, Switzerland.

Table 1. Detection of N-, O- and S-heterocyclic chemicals in polluted ground waters

Heteroatom	Chemical group or individual compounds	Use or likely origin of pollutant	Location	Reference
N	Pesticides: atrazine, bromacil, cyanazine, metribuzin, simazine	Agricultural practice	17 states	[15]
N	Methylpyridine (picoline)	Coal distillation; synthesis of pesticides, rubber, vinyl pyridine; solvent	Aquifer underlying municipal landfill, Norman, OK	[52,53]
O	(Alkylated) dioxanes/trioxanes, e.g., 1,3,5-trioxane; 2-methyl-1,3-dioxane; 2,4,6-trimethyl- 1,3,5-trioxane	Solvents	Aquifer underlying municipal landfill, Norman, OK	G. Smith[a]
S	Methylthiophene			
N	Pyridine; alkyl(C_1 to C_5) pyridines; e.g., *o*-, *m*- and *p*-picoline; azaarenes: quinoline, isoquinoline, alkyl(C_1,C_2)quinolines, methyltetrahydroquinoline	Coal gasification activities	Near underground gasification site, Wyoming	[54]
S	Alkyl(C_1 to C_4)thiophene, e.g., 2-methylthiophene			
O	Methylbenzofuran			
N	Alkyl(C_1,C_2)pyridine, e.g., picoline, caffeine	River water infiltrate to an aquifer	River Rhine, The Netherlands	[55]
O	Dibenzofuran	Creosote waste	Conroe waste site, Texas	[56]
S	Dibenzothiophene			
O	1,4-Dioxane	Solvent	Llangollen landfill	[57]
	Tetrahydrofuran	Solvent for polymers	Wilmington, DE	
N	Methyloxindole, 3-methylindole, nicotine, caffeine	Leachate	Sanitary landfill, Barcelona, Spain	[17]
O	Tetrahydrofuran	Leachate from municipal, domestic and industrial waste	3 municipal landfills in Connecticut	[58]
N	Alkyl(C_3)pyridine	Pollutants	Rhine River and infiltrate	[59]
S	Methylthiobenzthiazole			
N	Alkyl(C_1 and C_5)pyridines	Wastes from synthetic fuel industry	Drainage basin adjacent to oil shale facility, Rifle, CO	[47,60]
N	Methylindole	Leachate from municipal refuse	Ambt-Delden, The Netherlands	[61]
N	Polynuclear-azaheterocyclic compounds (azaarenes)	Leachates from creosote	Wood treatment plant, Pensacola, FL	[62]
		Leachates from coal tar	Coal tar distillation St. Louis Park, MN	[4]
O	Process water from 2-furaldehyde production	Deep (1,500–2,200 ft) underground injection facility	Belle Glade, FL	[63]

[a]Personal communication.

2-methyl-1 3-dioxane are also detected in ground water. Many of the ground water contaminants have monocyclic structures, but some, like the azaarenes, dibenzothiophene or dibenzofuran, are polynuclear.

Naturally occurring compounds also contribute to the burden of heterocyclic chemicals in ground water (Table 1). Albaiges and coworkers [17] identified caffeine, nicotine, 3-methylindole and methyloxindole as aquifer pollutants emanating from a

sanitary landfill. It is possible that the latter two chemicals might be metabolites of tryptophan. It is well known that the anaerobic biotransformation of tryptophan can result in the formation of indole and oxindole [18–20]. If tryptophan were degraded in a manner analogous to tyrosine, we would expect 3-methylindole and methyloxindole as metabolites.

The aerobic biodegradation of heterocyclic compounds has been reviewed [21–26] and has received far more attention than the metabolism of such compounds in the absence of molecular oxygen. Under the latter conditions, the catabolism of central metabolites such as nicotinic acid, purines, pyrimidines and tryptophan has also been reviewed [26,27]. However, the anaerobic metabolism of anthropogenic heterocyclic chemicals is virtually unknown [28].

The objective of our study was to compare the anaerobic biodegradation of model chemicals containing nitrogen, oxygen and sulfur ring atoms. The test compounds included pyridine, furan and thiophene derivatives that were unsubstituted or substituted with a carboxy or methyl group. The microbial metabolism of these substrates was evaluated with anoxic aquifer slurries incubated under sulfate-reducing and methanogenic conditions. Our findings demonstrate the potential for the anaerobic metabolism of heterocyclic substrates in a shallow aquifer and illustrate the need to understand the predominant ecological conditions for reliable predictions of a compound's subsurface transport and fate characteristics.

MATERIAL AND METHODS

Chemicals

All chemicals used were analytical grade or purchased at the highest available purity. Aldrich Chemical Co. (Milwaukee, WI) supplied furan, 2-methylfuran, 2-furoic acid, 2-, 3- and 4-picoline, thiophene, 2- and 3-methylthiophene and 2-thiophene carboxylic acid. Pyridine was purchased from Fisher Scientific Co. (Pittsburgh, PA) and nicotinic acid was obtained from Sigma Chemical Co. (St. Louis, MO).

Sampling and incubation of aquifer slurries

Aquifer solids and site water were sampled from two well-characterized sites adjacent to the Norman municipal landfill as previously described [29]. The sampling was conducted in late spring. The water table at the methanogenic site was 1.35 m below the surface and had a temperature of 18.3°C, a pH of 6.8, a total organic carbon content (TOC) of 288 mg/L and a sulfate concentration of less than 0.1 mM. The water table at the sulfate-reducing site was 1.15 m below the surface and had a temperature of 15.6°C, a pH of 7.2, a TOC of 14.4 mg/L and a sulfate concentration of 2.1 mM. Aquifer samples were transported to the laboratory and placed in an anaerobic glove box where 50 ± 1 g aquifer material and 50 ml site water were aseptically transferred to sterile glass serum bottles (160 ml). The bottles were closed under an atmosphere of N_2/CO_2 (80/20) and reducing conditions were controlled by the addition of Na_2S to reach an initial concentration of 1 mM. Filter-sterilized resazurin (0.0002%) was added as a redox indicator. The bottles were sealed with butyl rubber stoppers and crimped with aluminum seals. The bottles were stored for at least 5 d prior to the addition of the heterocyclic test compounds.

Ten-millimolar anaerobic stock solutions of all heterocyclic chemicals in distilled water were filter sterilized through 0.2-μm membrane filters. Stock solutions of the more hydrophobic methylthiophenes contained 1 ml/L methanol to facilitate their dissolution. All heterocyclic model compounds were aseptically added to aquifer slurries to reach an initial concentration of about 0.2 mM. This concentration was easily measured by the analytical techniques employed and allowed us to compare the level of sulfate reduction and methanogenesis relative to substrate-unamended controls. The experiments were conducted in duplicate, and autoclaved aquifer slurries (121°C, 15 lb/in^2, 180 min) served as controls. The biodegradation of each substrate was tested individually. The bottles were incubated in the dark at room temperature. One-ml samples of the aquifer slurries were aseptically and anaerobically removed using a syringe and needle immediately after the addition of the substrates and periodically thereafter during the course of the experiment. The samples were stored at −10°C until analyzed for parent substrate depletion.

Substrate and metabolite analysis

The samples from the aquifer slurries were thawed and centrifuged (5 min, 8,000 *g*) prior to reversed-phase high-pressure liquid chromatography (HPLC) of the supernatant using a C18 column. The equipment used for this analysis has been previously described [30,31]. A mixture of acetonitrile and acetate buffer (50 mM, pH 4.5) was used as the mobile phase at a flow rate of 1.5 ml/min. Compound detection was by UV absorbance with a variable-wavelength spectrophotometer (Beckman model 165). The analytical conditions (aceto-

nitrile/buffer ratio, wavelength in nm, retention time in min) for each compound are: pyridine (9/6, 255, 3.5), 2-picoline (9/6, 255, 5.7), 3-picoline (9/6, 255, 5.2), 4-picoline (10/5, 255, 5.8), nicotinic acid (10/5, 255, 4.8), furan (6/9, 212, 8.9), 2-methylfuran (7/8, 228, 9.6), 2-furoic acid (0/15, 248, 4.0), thiophene (7/8, 238, 6.2), 2-methylthiophene (6/11, 238, 4.0), 3-methylthiophene (9/6, 238, 5.9), and 2-thiophene carboxylic acid (0/15, 238, 8.3). The injection loop size was 100 μl and the detection limits for the picoline, furan and thiophene compounds were 10 to 25 μM, 1 to 5 μM and 1 to 5 μM, respectively. External standards were employed in all analyses.

Methane and sulfate analysis

The headspace concentration of methane in the serum bottles was determined by flame ionization GC as previously described [32] or with a Varian model 3300 gas chromatograph using a 1.8 m × 0.32 cm stainless steel column packed with 80/100 mesh poropack Q. Nitrogen was used as a carrier gas at a flow of 30 ml/min. The temperature of the injector, column and detector were 100, 105 and 120°C, respectively. External standards were employed and kept in stoppered glass serum bottles. Concentrations of methane in the headspace of methanogenic incubations were typically on the order of a few percent. Small pressure changes were not accounted for in the calculation of the methane formed.

The aqueous phase samples of the aquifer slurries were prepared as reported above for the substrate analysis and analyzed for sulfate by anion exchange HPLC. The mobile phase (4.0 mM potassium hydrogen phthalate, pH 5.0) was pumped at 2.5 ml/min using an LDC/Milton Roy minipump (model 396) through an anion exchange column (Vydac, 250 × 4.6 mm, Alltech Associates, Inc., Deerfield, IL). Sulfate had a retention time of 6.3 min and was detected with a Wescan (Santa Clara, CA) conductivity detector (model 213-505). The detection limit was at least 0.1 mM.

Stoichiometry of methane formation

Assuming complete mineralization of the heterocyclic chemicals, we used the general Buswell equation [33] to calculate the expected amount of methane:

$$C_nH_aO_bN_cS_d + (n - a/4 - b/2 + 7c/4 + d/2)\,H_2O \rightarrow (n/2 - a/8 + b/4 - 5c/8 + d/4)\,CO_2 + (n/2 + a/8 - b/4 - 3c/8 - d/4)\,CH_4 + cNH_4HCO_3 + dH_2S.$$

For heterocyclic acids, the protonated form was used for the calculation. Biodegradation rates and the stoichiometry of methane formation for the test chemicals are presented in the results.

The disappearance of the model compounds was measured by HPLC to calculate the μmol of substrate depleted in the serum bottles (60 ml liquid volume). This value was then multiplied by the expected CH_4 stoichiometry (moles CH_4/mol substrate) to obtain the expected amount of CH_4 (μmol) in the aquifer slurry due to the mineralization of the parent compound. We then accounted for the distribution of methane between the headspace and liquid phases of the serum bottles (Henry coefficient = 27.2, [34]) and the volume of gas and liquid (84/60 ml). We calculated that 97.4% of the methane partitioned to the headspace of the bottles. The total amount of methane formed in the serum bottles was corrected for the amount produced in substrate-unamended controls. The percent CH_4 recovery was calculated from the amount of CH_4 measured divided by the amount of CH_4 expected.

RESULTS

Biotransformation under sulfate-reducing and methanogenic conditions

The susceptibility of 12 model heterocyclic compounds to anaerobic biodegradation was screened by comparing substrate concentrations in sterile and nonsterile aquifer slurries obtained from two spatially distinct sites. Sulfate-reducing conditions predominated at one aquifer site while the other was primarily methanogenic [29]. Prior GC/MS analysis of the chemical constituents in the ground water from this aquifer confirmed the presence of nitrogen, oxygen and sulfur heterocyclic pollutants (Table 1).

The biodegradation of the test heterocyclic compounds was indicated by the amount of parent substrate remaining at one, three and eight months of incubation relative to a sterile control (Table 2). After one month, nicotinic acid, 2-furoic acid and furan disappeared under sulfate-reducing conditions while only the former two compounds were removed under methanogenic conditions. Eight of the 12 test compounds were at least partially degraded (>25% removed) under sulfate-reducing or methanogenic conditions by the end of the experiment (Table 2). Thiophene and methylthiophenes exhibited a relatively rapid abiotic loss (>80%/month) from both sterile and nonsterile incubations, possibly due to volatilization. Consequently, these compounds could only be followed for the first three months. Within this time frame,

Table 2. Disappearance of heterocyclic model compounds in sulfate-reducing (SR) and methanoganic (M) aquifer slurries

Heterocyclic model compound	Substrate remaining (%)[a]					
	SR aquifer slurries			M aquifer slurries		
	1 month	3 months	8 months	1 month	3 months	8 months
Pyridine	99	80	<5	115	115	<5[b]
2-Picoline	97	46	<8	105	107	97
3-Picoline	89	103	74	101	102	101
4-Picoline	75	47	65	97	93	<10
Nicotinic acid	<6	[c]	[c]	<6	[c]	[c]
Furan	11	[c]	[c]	102	58[b]	<3[b]
2-Methylfuran	112	29[b]	34[b]	111	106	102[b]
2-Furoic acid	<1	[c]	[c]	<1	[c]	[c]
Thiophene	104	122	[d]	100	111	[d]
2-Methylthiophene	90	90	[d]	93	145	[d]
3-Methylthiophene	98	112	[d]	93	96	[d]
2-Thiophene carboxylic acid	102	109	95	115	104	20

[a]Values represent the average substrate concentration in nonsterile replicates relative to sterile controls. The initial substrate concentrations were in the range of 0.14 to 0.25 mM. The standard deviation of the substrate concentrations between replicates was <0.01 mM at the beginning of the experiment and <0.02 mM during the course of the incubation if not indicated differently.
[b]Only one nonsterile replicate showed substrate disappearance.
[c]Reamended following complete substrate depletion.
[d]Abiotic loss precluded extended incubation times.

no biotic loss of the thiophene and methylthiophenes could be measured. The substrates 3-picoline and 2-methylfuran were only partially transformed in eight months; about 26 and 66% of 3-picoline and 2-methylfuran, respectively, were degraded under sulfate-reducing conditions (Table 2).

In general, the oxygen and nitrogen heterocyclic compounds were more amenable to anaerobic biodegradation than those containing a sulfur heteroatom. Table 2 shows that the concentrations of the tested pyridine compounds decreased significantly in the sulfate-reducing incubations within three months. In methanogenic incubations, somewhat longer adaptation periods were necessary. Similarly, furan and 2-furoic acid were rapidly depleted and over 70% loss of 2-methylfuran was observed within three months. However, none of the sulfur heterocycles were transformed within the same incubation period.

The effect of various substituent groups on the anaerobic biodegradation of heterocyclic structures was studied. With the exception of thiophene carboxylic acid under sulfate-reducing conditions, all carboxylated heterocyclic compounds were anaerobically biodegraded under both redox conditions. The unsubstituted heterocyclic chemicals were less amenable to anaerobic decay than the carboxylated derivatives, but generally more readily degraded than the methylated analogues. For instance, the concentrations of 2-furoic acid, furan and 2-methylfuran were >99, 88 and 0% depleted, respectively, within one month under sulfate-reducing conditions (Table 2). A similar comparison with the same conclusion can be made for these compounds incubated for three months under methanogenic conditions (Table 2). The degradation pattern of the nitrogen-containing test compounds presented a slightly more complex picture. Nicotinic acid was the most rapidly depleted compound under both redox conditions. The complete removal of nicotinic acid within one month was observed, whereas pyridine was depleted under the detection limit after a longer incubation period of eight months. The anaerobic biodegradation of methyl pyridines (picolines) exhibited a dependence on the position of the substituent, the redox conditions and the inoculum source. Complete removal of 2-picoline and 4-picoline was demonstrated under sulfate-reducing and methanogenic conditions, respectively (Table 2). The loss of 3-picoline never exceeded 30% in either of the aquifer incubations.

Three of the test compounds—furan, 2-furoic acid and nicotinic acid—were repeatedly reamended to aquifer slurries following their removal from nonsterile incubations. We found that each substrate addition was completely removed within one month from nonsterile aquifer slurries but not from sterile controls. An example of this is shown

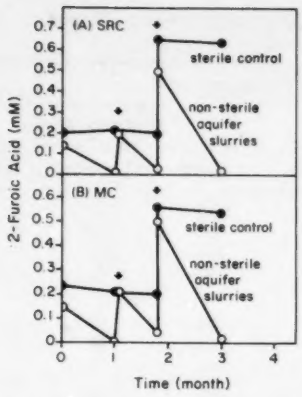

Fig. 1. Microbial removal of 2-furoic acid from aquifer slurries obtained from (A) a sulfate-reducing (SR) and (B) a methanogenic site (M). Arrows indicate the reamendment of parent substrate to the nonsterile samples and/or autoclaved control.

for 2-furoic acid under both sulfate-reducing and methanogenic conditions (Fig. 1). Figure 1 also demonstrates that an increase in substrate concentration to 0.5 mM still resulted in rapid substrate depletion relative to sterile controls.

Effect of substrate concentration on biodegradation

The influence of substrate concentration on the anaerobic biodegradation of a model heterocyclic substrate was tested by amending the aquifer slurries from both sites with various levels of 2-picoline. Initial substrate concentrations of 50, 200, 500 and 2,000 μM resulted in the disappearance of 100, 100, 89 and 25% of 2-picoline, respectively, following an eight-month incubation period in the sulfate-reducing incubations. Over the same time period, the sulfate concentration dropped to 0.21, 0.12, 0.10 and <0.10 mM in aquifer slurries receiving increasing starting concentrations of 2-picoline. The sulfate level in the substrate unamended controls dropped to 0.2 mM over the same time period. Further, the unamended control-corrected headspace CH_4 concentration increased 0.15 (0.23 mmol), 0.22 (0.34 mmol), 0.92 (1.43 mmol) and 1.54% (2.40 mmol), respectively, as a function of increasing substrate concentration. These results indicate that the more substrate the slurries received the more sulfate was depleted and higher levels of methane were formed. Such data suggest that during the incubation at least a partial shift in the flow of carbon and energy from sulfate reduction towards methanogenesis occurred as sulfate was depleted. At lower substrate concentrations, a complete loss of 2-picoline was observed; at the higher concentrations only a partial loss could be measured. The amount of substrate depletion (μmol per bottle) was about equal in aquifer incubations receiving the higher concentrations of 2-picoline (500 and 2,000 μM). At the higher substrate levels, biodegradation may be limited by the availability of sulfate or other nutrients. Presumably, the additional headspace methane resulted from the mineralization of a picoline metabolite or from the methanogenic fermentation of endogenous organic matter following sulfate depletion.

Parallel experiments were also conducted with 2-picoline in methanogenic aquifer slurries. However, 2-picoline was not transformed at any of the test concentrations. Headspace methane concentrations were not different in substrate-amended or unamended controls. These results suggest that 2-picoline was not toxic to the indigenous microflora but that the requisite microbial communities were simply absent or inactive under methanogenic conditions.

Carbon mass balances

Mass balance studies were conducted with methanogenic aquifer slurries. Sulfate was not detected (<0.1 mM) in the aquifer slurries used for this study. No increase in CH_4 formation was observed in incubations that were unable to biologically transform the model substrates. The amount of CH_4 that could theoretically be expected in the aquifer slurries was calculated and compared to the actual amount of substrate converted to CH_4 (Table 3). This analysis suggested that pyridine, 4-picoline, nicotinic acid and 2-thiophene carboxylic acid could be completely mineralized. The amount of methane recovered for these compounds varied from 47 to 67% of the expected amount (Table 3). Methane recoveries from oxygen heterocyclic chemicals were much smaller (e.g., 2-furoic acid) or not detectable (e.g., furan).

To verify that 4-picoline was indeed mineralized, we attempted to transfer 4-picoline-degrading bacteria by placing 10 ml liquid from an actively degrading methanogenic slurry to unamended aquifer slurries (triplicates). Although amended at a higher concentration (0.5 mM), the substrate was more rapidly metabolized than it was in the initial experiment. Within three months 94% of 4-picoline was degraded and 83.3 (SD = ±14.6) μmol methane was formed over unamended controls. This was 84.4 (SD = ±14.8) % of the theoretically expected amount of methane from this substrate.

Table 3. Methane formation from heterocyclic chemicals in aquifer slurries from the methanogenic site

Heterocyclic compound	Basic chemical structure	R	μmol substrate depleted[a]	Stoichiometry (mol CH_4 per mol substrate	μmol CH_4 expected[b]	μmol CH_4 formed	% recovered as CH_4
Pyridine		H	12	2.75	33	15.4	46.7
4-picoline		CH_3	12	3.5	42	28.1	66.9
3-picoline		CH_3	[c]	3.5	0	<4	[c]
2-picoline		CH_3	[c]	3.5	0	<4	[c]
Nicotinic acid		COOH	54	2.75	148.5	82.7	55.7
Furan		H	24	2.25	54	<4	<7
2-methylfuran		CH_3	[c]	3	0	<4	[c]
2-furoic acid		COOH	54	2.25	121.5	31	25.5
Thiophene		H	[c]	2.25	0	<4	[c]
2-methylthiophene		CH_3	[c,d]	3	0	<4	[c,d]
3-methylthiophene		CH_3	[c,d]	3	0	<4	[c,d]
2-thiophene carboxylic acid		COOH	8.9	2.25	20	11.3	65.5

[a]Calculated from Table 2 for the eight-month period.
[b]Relative to nonamended controls.
[c]No significant transformation of the parent substrate.
[d]While methane was formed, it was not in excess of that expected from the methanol carrier.

DISCUSSION

Most of the tested heterocyclic model compounds were biotransformed in aquifer slurries obtained from either the sulfate-reducing or the methanogenic sampling site. The substitution of a heterocyclic compound with a carboxy group rendered the chemical more amenable to microbial attack compared to methylated or unsubstituted analogues. This was true for chemicals containing a nitrogen, oxygen or sulfur heteroatom incubated under either of the employed redox conditions.

These observations are similar to findings made with homocyclic compounds. The anaerobic catabolism of carboxylated benzene (benzoate) is rapid in the presence of many different electron acceptors and has been well known for several decades [27,35]. However, the methylated derivative (toluene) or the unsubstituted homocyclic compound (benzene) are much more recalcitrant when anoxic conditions prevail. The anaerobic catabolism of such compounds could only recently be conclusively shown under denitrifying and methanogenic conditions [36–38].

The primary degradative step for heterocyclic compounds often involves the hydroxylation of the ring with H_2O. Most commonly, this substitution occurs at the position adjacent to the heteroatom as was suggested for nicotinic acid [39], purines [26], pyrimidines [26], quinoline [28] and indole [27]. The initial hydroxylation of a heterocyclic compound with H_2O was also shown to occur in the presence of molecular oxygen [28,40]. A similar reaction may also be crucial for the anaerobic biotransformation of homocyclic chemicals. One laboratory has been able to demonstrate the hydroxylation of the benzene nucleus with water as a coreactant [38]. A polar functional group on a homocyclic as well as a heterocyclic ring facilitates the anaerobic metabolism of the respective structures. Aromatic methyl substituents can be oxidized under aerobic or anaerobic conditions eventually to carboxylated derivatives with H_2O serving as the initial coreactant [31,41]. This reaction seems to be a crucial step in the anaerobic biodegradation pathway of several homocyclic chemicals, including *p*-cresol [31,42], *o*-cresol [43] and toluene [37]. The anaerobic degradation pathway of a methylated heterocyclic compound is not known. However, it may be that the initial hydroxylation of the aryl methyl group occurs prior to ring hydroxylation. If true, the alkyl group oxidation might presumably represent the rate-limiting step in the catabolic pathway.

Recently, Pereira and coworkers discovered novel biotransformation reactions of azaarene chemicals in anoxic ground waters and in methanogenic enrichment cultures [44]. They reported that several azaarenes (e.g., quinoline, isoquinoline, 4-methylquinoline) were initially hydroxylated and subsequently metabolized in a series of N-, O- or C-methylation reactions. The accumulation of the methylated products in the aquifer and methanogenic digestors, as well as the lack of methane for-

mation in azaarene-amended digestors, suggests that such materials are recalcitrant under the studied conditions [44]. These results support our finding that methylated heterocyclic chemicals are not easily biotransformed under anaerobic conditions.

The anaerobic bacterial metabolism and growth on purines and pyrimidines is known for various fermentative strains, including several clostridia [26]. Bak and Widdel [45] demonstrated that sulfate-reducing microorganisms could utilize nitrogen heterocyclic compounds like quinoline, pyridine, indole and skatole (3-methylindole) as sole sources of carbon and energy. Further, two research groups found methane formation from indole in anaerobic incubations [20,46]. These investigations indicate that complete mineralization of certain heterocyclic compounds under sulfate-reducing or methanogenic conditions is possible. These observations and our own studies confirm that both naturally occurring and anthropogenic heterocyclic materials can be mineralized under anaerobic conditions.

This report is the first to show the mineralization of pyridine and 4-picoline (4-methylpyridine) under methanogenic conditions. Although we also observed the anaerobic degradation of alkylated pyridines (picolines) in aquifer slurries, such compounds have to be considered recalcitrant because (a) they exhibit long lag times prior to disappearance, (b) not all isomers were anaerobically biotransformed and (c) the biodegradation activity was not observed under both redox conditions. Since alkylated heterocyclic chemicals are frequently detected as ground water contaminants (Table 1), it may be that longer alkyl groups and muitiple methyl substituents hinder the anaerobic destruction of such materials. Our results notwithstanding, there is at least one report that suggests that even larger molecular weight alkyl pyridines may be subject to removal mechanisms in anaerobic ground waters [47]. However, the contribution of the indigenous microflora to these removal mechanisms remains unclear.

Relative to nitrogen-containing heterocyclic compounds, much less is known about the anaerobic metabolism of chemicals containing oxygen or sulfur as the heteroatom. Although the microorganisms in methanogenic aquifer slurries did transform furan and 2-furoic acid, carbon mass balances indicated that methane formation from the two substrates was less than expected. Another study suggested that acclimated anaerobic sludges can anaerobically convert 2-furaldehyde to methane [48]. Recently, a *Desulfovibrio* sp. was isolated from a methane-producing fermentor that received sulfite evaporator condensate [49]. The strain transformed 2-furaldehyde mainly to acetate and possibly used sulfate as an electron acceptor. Thus, the metabolism of similar heterocyclic compounds can be coupled with either methanogenesis or sulfate reduction.

We also found that 2-thiophene carboxylic acid could be mineralized under methanogenic conditions but no transformation of the methylthiophenes was observed. In this respect it is interesting to note that Tanaka and coworkers [50] found that benzoate-grown and washed cell suspensions of a photosynthetic bacterium were capable of reducing 2-thiophene carboxylic acid to the tetrahydro derivative. Further, anaerobic cultures of thiophene-degrading microorganisms could also be obtained from sludges collected near an oil well [51]. Cell-free extracts of the microbial cultures released H_2S from thiophene in the presence of methyl viologen and H_2. We are not aware of other systematic studies designated to examine the effect of alkyl substituents on the anaerobic biodegradability of thiophene or furan compounds.

While our studies show that important classes of heterocyclic ground water contaminants are susceptible to anaerobic microbial destruction, the degradation pathways employed by the requisite microorganisms have still to be elucidated. Further, our studies show that the nitrogen and oxygen heterocyclic compounds were more susceptible to anaerobic biodegradation than those compounds containing a sulfur heteroatom. Carboxy substituted compounds were anaerobically metabolized more readily than unsubstituted or methylated analogues. More fundamental microbiological and biochemical information is necessary to understand the transport and fate characteristics of nitrogen, oxygen and sulfur heterocyclic pollutants in anoxic aquifers.

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